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The acquired immune deficiency syndrome (AIDS) is the clinically apparent preterminal stage of a prolonged infection with the human immunodeficiency virus (HIV), most often manifested as Pneumocystis carinii pneumonia, Kaposi's sarcoma, or other opportunistic infections or malignancies. AIDS is by definition a clinical syndrome diagnosis.

This article deals exclusively with the diagnosis of HIV infection, not with AIDS, and reflects the author's belief that quality medical care demands a precise etiologic diagnosis. A United States Presidential Commission recently stated that "The term AIDS is obsolete." Nowhere is this more true than in the diagnostic laboratory.

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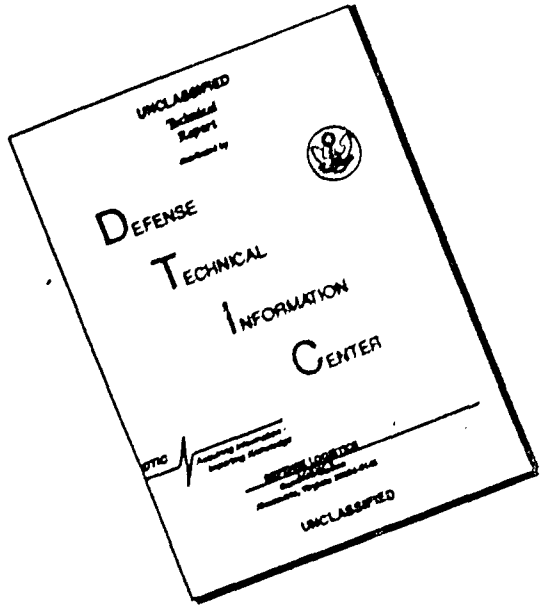

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Laboratory Diagnosis of Human Immunodeficiency Virus Infection

*Donald S. Burke, MD**

The acquired immune deficiency syndrome (AIDS) is the clinically apparent preterminal stage of a prolonged infection with the human immunodeficiency virus (HIV), most often manifested as *Pneumocystis carinii* pneumonia, Kaposi's sarcoma, or other opportunistic infections or malignancies. AIDS is by definition a clinical syndrome diagnosis.

This article deals exclusively with the diagnosis of HIV infection, not with AIDS, and reflects the author's belief that quality medical care demands a precise etiologic diagnosis. A United States Presidential Commission recently stated that "The term AIDS is obsolete."[98] Nowhere is this more true than in the diagnostic laboratory.

THE VIRUS AND CLINICAL COURSE

HIV type 1 (HIV-1), a retrovirus of the Family Lentiviridae, is an enveloped, RNA-containing virus with a diameter of 80 to 120 nm.[44] Two glycoproteins are exposed on the viral membrane, gp120 and gp41.[5] The gp120 (external membrane glycoprotein) includes the amino acid sequences responsible for virion binding to the CD4 molecule on the surface of T-helper cells. The gp41 (transmembrane protein) is thought to anchor gp120 to the virion surface and to be involved in fusion of the viral and target cell membranes. Within the membrane is a nucleocapsid in the shape of a truncated cone.

Two nonglycosylated proteins, p24 and p17, are important internal structural proteins.[36] Reverse transcriptase, the enzyme protein that synthesizes DNA from the virion RNA template, is carried in the virion core along with the RNA.[37]

The genome of HIV contains at least nine distinct genes that encode polypeptides. Three of these, the gag, pol, and env genes, code for the structural proteins.[48] The gag (group antigen) gene codes for the core

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proteins p17 and p24, as well as their common precursor p55; the pol (polymerase) gene codes for proteins with proteinase, reverse transcriptase, and endonuclease activity; and the env gene codes for the external membrane (gp120) and transmembrane (gp41) proteins. In addition to the structural genes, there are at least six other genomic open reading frames that encode proteins which are thought to be involved in regulation of virus growth through activation or suppression of transcription or translation of the viral structural genes (Table 1).

Although HIV was initially thought to be primarily an infection of CD4 positive T-helper lymphocytes, it is now clear that cells of the monocyte/macrophage lineage can support virus growth in vitro and in vivo.[46,49] Some continuous cell lines derived from other organs, such as intestinal epithelial cells, can also be permissive for HIV. Cell activation and proliferation accelerate virus replication.

In the hours and days immediately following HIV infection, the virus probably first gains entry into and replicates in mononuclear cells in the mucous membranes lining the genital tract and in mononuclear cells and follicular dendritic cells of local lymph nodes.[16] Within one to a few weeks HIV appears free in the plasma and within circulating mononuclear cells. There is no detectable antibody response during this so-called seronegative viremic phase or "window."[57] HIV antibodies typically first appear in the blood 4 to 12 weeks after initial infection, but rarely the "window" and may last up to 6 months or longer. As antibodies to the env and gag and then pol gene products arise, viral antigens decline and disappear; virus isolation from the blood becomes more difficult.[30] The acute phase of infection is usually asymptomatic, but 20 per cent of patients may experience a nondescript illness characterized by low-grade fever, fine maculopapular rash, and diarrhea.[58,94] Severe illness during the acute phase of infection, manifested by fever and encephalopathy, is relatively rare.[25]

Table 1. *The Known Genes of HIV-1 and the Proteins Encoded by These Genes*

GENE NAME	FUNCTION
Structural Genes (Products are released with newly formed virions)	
GAG	Core proteins (p55, p24, p17)
POL	Viral enzymes (p64, p53, p31)
ENV	Envelope proteins (gp160, gp120, gp41)
Regulatory Genes (Products are confined to host cell)	
TAT	Transactivator
REV*	Regulator of virion protein expression
VIF†	Virion infectivity factor
NEF‡	Negative regulatory factor
VPR	? Unknown
VPU	? Unknown

Note that only the structural proteins are present in purified virion preparations. Purified virions are used in preparation of conventional Western blot strips; as a consequence, only antibodies to the virion structural proteins can be detected in conventional HIV-1 Western blot strips.

* Formerly named ART or TRS.

† Formerly named SOR.

‡ Formerly named 3′-ORF.

A prolonged phase follows in which the patient is seropositive but asymptomatic. Despite the absence of symptoms, the chronic HIV infection induces subtle and progressive immune dysfunction, which may be clinically manifest only as lymphadenopathy or which may be clinically totally silent.[53,82] Viral antigens can be demonstrated primarily in follicular dendritic cells. Over the next several years numerous immune defects appear, the most prominent being the progressive loss of CD4 positive T-helper lymphocytes. When the CD4 T-helper lymphocyte count drops below 100 per cubic mm, overt manifestations of HIV infection are imminent, typically in the form of opportunistic infections. Severe immunocompromise is accompanied by a decline in HIV antibodies, especially a pronounced loss of anti-gag, and a rise in HIV antigen.[54,82] During the late stage of infection, virus can again be easily isolated from blood.

ANTIBODY TESTS

SCREENING ASSAYS

Screening assays for detection of HIV antibodies have in common the properties of high sensitivity, ease of performance, rapidity, and low cost. In the United States the only assays presently available for screening are enzyme-linked immunosorbent assays (ELISAs), which use lysates of partially purified whole virions as a source of viral antigens for sensitization of a solid plastic surface (such as a microtiter plate well or a bead). HIV-specific human antibodies in the test serum specimen are bound to viral antigens on the plastic surface and detected with complexes of enzymes and animal antibodies that specifically recognize human immunoglobulins. Enzymatic activity bound to the surface is in turn determined with a soluble substrate that is cleaved and changes color. The resultant color change (change in optical density or OD) is directly proportional to the concentration of HIV antibodies in the test serum specimen. Whole virus lysate ELISAs were first licensed in early 1985 for the purpose of screening donated blood. These assays promptly found wide application as the initial step in algorithms designed for the diagnosis of HIV infections.

An HIV whole virus lysate ELISA should not be used as a "stand alone" test for diagnosing HIV. Although the specificity of this type of assay is quite good (on the order of 99.5 per cent for most commercial kits), some true negative samples do give reproducible falsely reactive results.[45,56,84] In most cases the cause of the false reactivity cannot be readily discerned, but associations with autoimmune diseases and with multiparity (among women) have been reported.[6,18] Depending on the prevalence of HIV in the population being screened, the predictive value of a repeatedly reactive ELISA may be greater than 90 per cent (for example, in a clinic for homosexual men in New York) or lower than 10 per cent (for example, in a blood bank in North Dakota). False reactivity is thought to be caused by antibody binding to cellular proteins that contaminate the purified virions. Some of the contaminating cellular proteins may actually be cell surface proteins that become incorporated

into the virion membrane during the process of viral budding from cell membranes.

More recently, screening assays based on agglutination of small particles (red blood cells or latex particles) have been developed.[102] In contrast to ELISAs, which require equipment for measurement of optical densities, agglutination reactions can be scored by the naked eye, a distinct advantage in a field setting. However, agglutination reactions can be difficult to interpret and require trained observers. Although precise data are not yet available, it seems unlikely that a subjectively scored agglutination test will ever achieve performance equal to the quantitatively scored ELISA. The same constraints that apply to the predictive value of a positive ELISA, will also limit the utility of agglutination reactions for diagnosing HIV. In most circumstances suboptimal specificity cannot be tolerated; therefore, a positive agglutination test should also be confirmed with an independent, and preferably more specific, assay method.

CONFIRMATORY TESTS

Despite the preference of some for the term supplemental test, this author is of the opinion that "supplemental" connotes a level of uncertainty which is not conveyed in the term "confirmatory." Since these assays are widely used to establish a firm diagnosis of HIV in the clinical practice of medicine, the term confirmatory seems to be more appropriate and, thus, is used in this article.

Western Blot

The Western blot, or immunoblot, is the most widely used confirmatory test for the detection of HIV antibodies.[95] As in the screening ELISA, the source of HIV antigens is partially purified whole virions harvested from in vitro cultures of infected human lymphocyte cell lines. Lysed virus is "vertically" electrophoresed through a sizing polyacrylamide gel, and the separated and banded viral proteins are "horizontally" electroblotted from within the gel onto the surface of nitrocellulose paper. The paper is cut into several strips, each of which has essentially identically located bands of viral proteins. (Although viral band positions on paper strips derived from a single sizing gel run are identically located, slight alterations in electrophoretic conditions from run to run may result in minor alterations in the migration distances of viral proteins from run to run. For this reason, interpretation of banding patterns is facilitated by testing of positive and negative control specimens using strips from the same run that are used for the unknown samples.) Note that contaminating cellular proteins in the virion preparation are also electrophoresed, transblotted, and distributed on the paper strips with the viral proteins. One paper strip is then used as the solid phase to detect antiviral antibodies using reagents and methods similar to the ELISA: binding of HIV-specific antibodies present in the test serum specimen to proteins on the Western blot strip eventuates in a color change at those locations (band) where the antibodies have bound.

Most Western blot strips prepared in the United States before 1987 lacked appreciable bands corresponding to the high molecular weight viral envelope proteins (gp120 and its precursor gp160).[24] Underrepresentation of these proteins was probably owing to several factors, especially loss of gp120 from the virion surface due to shearing during purification by ultracentrifugation, and by denaturing of antigenic activity during electrophoresis and transblotting. Since early 1987 blot preparation methods have been modified to ensure that the high molecular weight envelope bands can be clearly identified with most patient sera.

Strongly positive HIV Western blots show dark bands at positions corresponding to the all the major gag (p55, p24, and p17), pol (p64, p53, and p31) and env (gp160, gp120, and gp41) bands. Gag and pol bands are relatively narrow with sharp edges, whereas the glycosylated env bands tend to be broader with indistinct upper and lower margins (the gp160 and gp120 bands often overlap). Presence of the gag (p55) and env (gp160) precursor proteins on Western blots supposedly derived from purified virions suggests incomplete purification of virion from intracelluiar proteins, but the possibility that some structural proteins can be incorporated into intact virions without uniform cleavage has not been ruled out. Nonstructural regulatory HIV encoded proteins are separated from the virions during purification, and antibodies to these proteins are not identifiable on standard Western blots.

Western blots performed using antibody negative sera usually show no bands and are uniformly "snowy white" for their entire length. All nine viral structural proteins are present on the strip surface, but the bands remain colorless because no antibodies have bound. With some strongly reactive sera all nine bands are darkly stained. However, not all sera from definitely infected persons show reactivity with all nine HIV structural proteins on Western blot, and not all sera from definitely uninfected persons produce blank strips. For this reason, interpretive criteria must be used to evaluate the significance of various Western blot patterns.

Before 1987 (and before antibodies to gp160 and gp120 could be regularly visualized by blot), criteria *informally* presented by the U.S. Public Health Service were widely used (p24 or gp41, CDC; p24 and gp41, NIH) to define a positive Western blot. In U.S. Department of Defense HIV testing programs, somewhat more specific criteria were employed (p24 + p55 and/or gp41). However, it quickly became apparent that all of these criteria sets were less than perfectly specific. False positive gag p24 reactivity was a major problem, and some cases with falsely reactive bands at both p24 and p55 were identified.

Regrettably, there are still no U.S. nationally (or internationally) agreed upon criteria for Western blot interpretation. Several groups concerned with HIV serodiagnosis currently use different sets of interpretive criteria. All agree that a snow white, clean lane pattern is negative. However, there are major differences in the definition of positive blot (Table 2). Some criteria currently in use include:

1. The Association of State and Territorial Public Health Laboratory Directors and the Department of Defense: two or three of three bands among p24, gp41, gp120/160

Table 2. *Six Different Sets of Criteria for a Positive Western Blot*

YEAR	AGENCY	CRITERIA FOR POSITIVE WESTERN BLOT	
Pre Gp120–Gp160			
1984	CDC	24 or 41	
1985	NIH	24 and 41	
1985	DOD	(24 and 55) or 41	
Post Gp120–Gp160			
1987	ASTPHLD	24	
		41	2 of 3
		120–160	
1987	FDA	24	
		31	3 of 3
		41–120–160	
1987	Red Cross	15–24–55	
		31–53–64	3 of 3
		41–120–160	

2. The American Red Cross: at least one gag (p55, p24, p17), one pol (p64, p53, p31), and one env (gp41, gp120/160) band

3. The Food and Drug Administration (FDA; per the package insert in the licensed DuPont Western blot kit): three of three among p24, p31, (gp41 and/or gp120/160)

The estimated sensitivity and specificity of these Western blot interpretive criteria are presented in Figure 1. The old and now abandoned CDC criteria were highly sensitive but poorly specific. Conversely, current FDA criteria (on the package insert) are quite specific but show poor sensitivity.

By convention, all strips that do not meet the criteria for a positive Western blot but that do show one or more bands at molecular weights corresponding to HIV proteins are termed reactive but nondiagnostic or indeterminant. Up to 15 per cent of sera from normal noninfected persons when tested by the FDA licensed commercial Western blot (DuPont) show a reactive but nondiagnostic (indeterminant) pattern. Indeterminant Western blot patterns are found as commonly among specimens that are negative by screening ELISA as among specimens that are screening ELISA-reactive. Most indeterminant blots show only a weak gag band, most commonly at p17 or at p24.[24,77,97] Other single band, and occasionally multiple band, indeterminant blots are less frequent. When a new blood sample is drawn and immediately tested from a person with a recent indeterminant blot result, the repeat is usually completely negative. However, many normal persons do have repeatedly positive, "gag only" indeterminant Western blots.[22] The significance of an indeterminant blot is at present poorly understood. Many, especially those with weak and transient reactivity, are probably caused by technical errors of specimen contamination by pipette tip carryover or splashing. Rarely, an indeterminant blot signifies a specimen obtained early during seroconversion to HIV. Other intriguing possibilities exist, such as prior infection with an as-yet unidentified related retrovirus, or autoantibodies to antigenically related epitopes on normal human proteins, but these currently remain in the realm of theoretic possibilities.

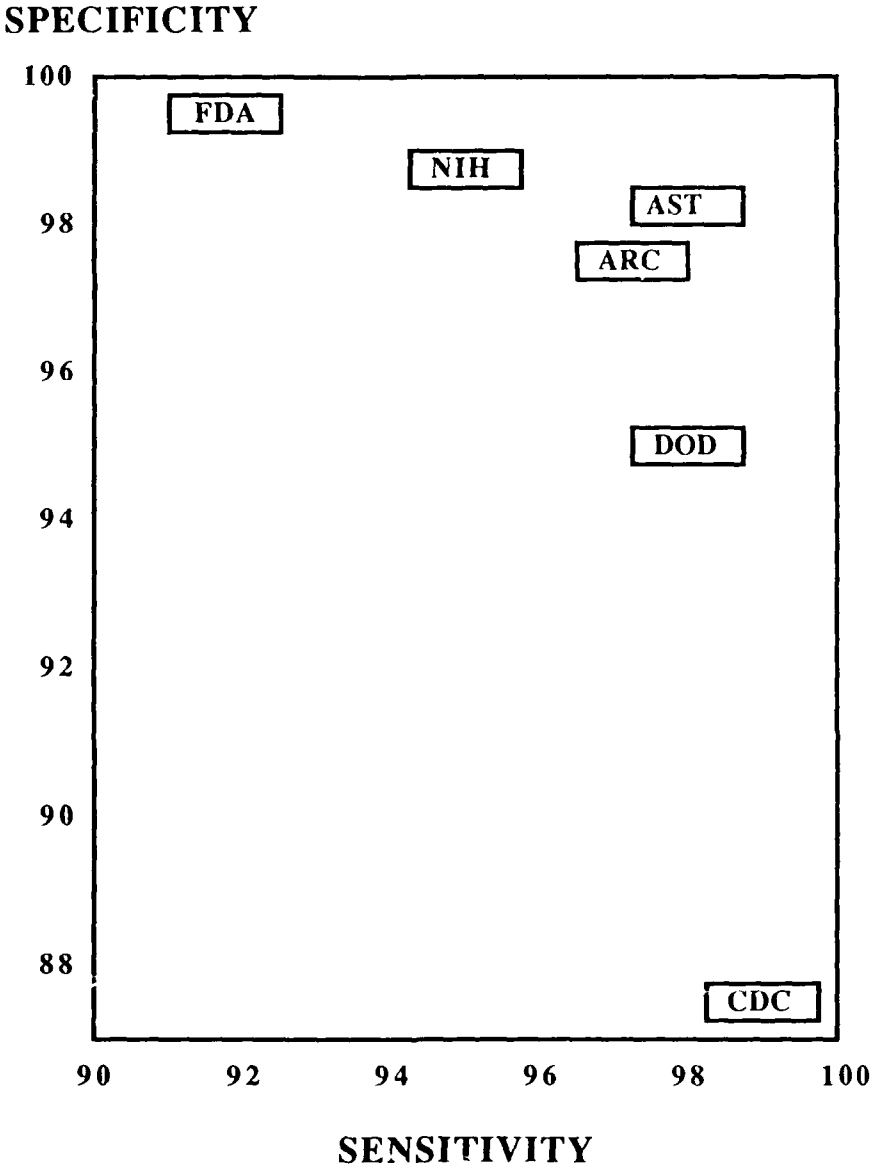

Figure 1. Sensitivity and specificity of various criteria for interpreting HIV-1 Western blot. True status of specimens determined by repeated Western blot testing, results of testing by antibody assays constructed from molecularly cloned and expressed envelope polypeptides, and results of radioimmunoprecipitation assays. For exact definitions of each of the six interpretive criteria shown see Table 2.

Approaches to further testing and reporting of indeterminant Western blot results are provided in detail in the discussion of screening in low prevalence populations.

Immunoassays Constructed from Antigens Produced Through Recombinant DNA Technology or by Chemical Synthesis

In the few short years since the first isolation of HTLV-IIIB and LAV, the complete or partial nucleotide sequence of the genomes of at least 17 HIV strains has been determined. The genes for the structural proteins have been located, and hypervariable, variable, and highly conserved nucleotide sequences have been identified within these genes.[17] This detailed genetic information has been used to construct a variety of ELISAs for detection of HIV antibodies in which molecularly cloned and expressed antigens or synthetic antigens are used in place of the whole virus lysate antigens.

The first molecularly defined antigens used in construction of diagnostic ELISAs were selected largely by guesswork. Since it was known that antibodies to the env gene proteins appear early during seroconversion and persist until death, various constructs which included conserved regions of the env gene were cloned and expressed in *Escherichia coli*. This was done without knowledge of exactly which regions of the env gene were immunodominant. Conserved sequences (nucleotide sequences found essentially identical in all known HIV strains) were emphasized to ensure that all human sera would be reactive with the expressed antigen. One such ELISA produced from a molecularly cloned and expressed antigen spanning the carboxyl-terminal third of gp120 and the amino-terminal half of gp41 (CBre3, produced by Cambridge Biosciences, Inc.) was exhaustively evaluated and found to be comparable or superior to the Western blot in its sensitivity and specificity.[20] Because the gene was expressed in *E. coli*, the problem of false reactivity with lymphocyte proteins was eliminated. (The analogous problem of false reactivity with contaminating *E. coli* proteins was minimized by antigen purification; removal of *E. coli* proteins from the antigen was essentially complete, as proven by absence of reactivity of sera from patients convalescent from *E. coli* sepsis.) The CBre3 env ELISA has been used extensively as a second confirmatory test in Department of Defense HIV testing programs with excellent results. Other assays constructed from molecularly cloned and expressed HIV antigens have been produced and are currently undergoing clinical trials. Various strategies have been used to construct assays: only a single antigen (env) in an ELISA format, a single antigen (env) in a dot-blot or slot-blot format, two fused antigens (env and gag) in an ELISA format, multiple antigens (gag, pol, and env) in separate wells in an ELISA format, or multiple antigens (gag, pol, and env) in a multi-slot-blot "pseudo-Western blot" format.

To date none of these assays, including the CBre3 env assay, has been licensed by the FDA. However, because the performance of this type of assay appears to be excellent, safety concerns during antigen production are nil, and costs of antigen preparation can be lowered, it seems likely that molecularly cloned and expressed antigens will rapidly replace virion derived antigens in the construction of diagnostic assays

for HIV antibodies. Whether in the future assays constructed from molecularly cloned and expressed antigens will be used predominantly as confirmatory assays or as screening assays will be determined largely by cost considerations.

In contrast to molecularly cloned and expressed antigens, which are typically several hundred amino acids long and contain several epitopes, synthetic peptides are usually only 15 to 30 amino acids in length and encompass only one or two epitopes. Intensive efforts to pinpoint immunodominant epitopes (both for purposes of vaccine development as well as for diagnostic assay development) have resulted in the identification of several synthetic peptides that may serve as antigen surrogates.[29,51,89,90]

One epitope, located at the position corresponding to amino acids numbers 582 to 600 on env gene, is clearly the most immunodominant epitope identified to date. Essentially all seropositive persons in North America have antibodies that recognize and bind to this peptide. Indeed, on the basis of available data, it appears that all molecularly cloned and expressed antigens that include this epitope are excellent for use in diagnostic assay construction, whereas those that do not include this epitope have suboptimal sensitivity. Minor differences in nucleotide sequence between HIV strains from Africa and those in the United States render this peptide less than perfectly sensitive for detecting seropositive Africans.[50] It would seem imprudent to base a diagnostic test for HIV on a single epitope; should the strains prevalent in the United States mutate at this site, a disastrous loss in test sensitivity could ensue. However, a diagnostic assay constructed from a "cocktail" or two or more synthetic peptides is a realistic alternative. More likely is the use of single epitope synthetic peptide antigens in the construction of assays for determining stage and prognosis.

Indirect Immunofluorescent Antibody Test (IFA)

Some laboratories with extensive IFA experience have found this technique useful for confirming the presence of HIV antibodies.[67,72,96] Mixtures of approximately equal numbers of HIV-infected and uninfected cells are spotted onto slides, reacted with dilutions of the test serum specimen, and stained. Negative samples produce no fluorescence. False positive reactions are differentiated from true positive reactions by counting the percentage of cells showing fluorescence: 100 per cent with falsely reactive specimens and 50 per cent with truly reactive specimens. When appropriately controlled, the assay is reported to show sensitivity and specificity at least comparable to the Western blot.

Radioimmunoprecipitation Antibody (RIPA) Test

Some research-oriented laboratories use the radioimmunoprecipitation antibody test to confirm the presence of HIV antibodies.[12,28,93] In this technique, radiolabeled amino acids (such as S-35 cysteine) are added to infected cell cultures. The labeled amino acids are incorporated into polypeptides that are being actively translated. By selection of the correct labeled amino acid and correct time interval from label addition, intrinsically labeled viral proteins with high specific activity can be ob-

tained. Patient sera are added to the clarified labeled cell lysate, and antigen-antibody complexes are precipitated from solution. The resultant precipitate is electrophoresed over a polyacrylamide sizing gel, and bands are detected by autoradiography. Interpretive criteria for RIPAs vary from laboratory to laboratory, depending on which viral proteins are preferentially labeled. In the author's laboratory conditions were selected to optimally label the large envelope glycoproteins gp160 and gp120; the presence of bands at both gp160 and gp120 is necessary and sufficient for a positive diagnosis. The sensitivity and specificity of the technique have not been formally evaluated. There is some evidence that absence of antibodies to the carboxyl-terminal portion of gp120, as detected by RIPA, is associated with disease progression.

Other Diagnostic HIV Antibody Tests

In addition to the screening ELISA, Western blot, assays based on molecularly cloned and expressed or synthetic antigens, IFA, and RIPA, a number of other assays have been used to detect HIV antibodies. Most notable among these are the IgM capture assay and assays to detect HIV antibody synthesis in vitro.

Efforts to develop IgM capture assays for HIV antibodies have met with only marginal success.[15,26,43,66,74,76,81] In contrast to other chronic viral illnesses such as hepatitis B, the signal to noise (P/N) ratio is typically quite low, even during seroconversion; indeed many groups have failed to detect specific antibodies with this technique. Perhaps IgM assays using defined synthetic epitopes may show improved sensitivity.

The problem of differentiating between passively acquired versus actively produced antibodies in neonates born to HIV-infected mothers has led to the development of a technique for detecting HIV antibody synthesis in vitro in cultures of peripheral blood leukocytes from the neonate.[8,35] The technique, involving culture of patient cells, is cumbersome but shows promise.

"FUNCTIONAL" ANTIBODY TESTS

In contrast to the assays noted earlier, which simply detect antibodies that bind to HIV antigens, several methods have been reported for measuring antibody activities thought to have in vivo significance. Among these are virus neutralization, reverse transcriptase inhibition, and antibody-dependent cell-mediated cytotoxicity. Although one or several of these assays may eventually be proven mechanistically relevant to protection against infection or protection against disease progression, there is at present no compelling evidence of such a mechanistic relationship.

VIRUS ISOLATION

Because isolation of HIV from blood or other clinical specimens is time consuming, expensive, and potentially dangerous, virus isolation cannot be considered a standard diagnostic test for HIV. However, virus

isolation can yield useful information in unusual cases and in clinical studies.[10,32,38,55,92] All cultures must be handled with strict adherence to proper biocontainment protocols.

The conventional technique is to cocultivate patient peripheral blood mononuclear cells (PBMCs), with interleukin 2 (IL-2) and phytohemagglutinin-stimulated normal donor PBMCs, conditions that selectively stimulate activation and proliferation of CD4-positive lymphocytes. Cell culture supernatants are periodically monitored for appearance of HIV antigens and reverse transcriptase (RT) activity. The minimum number of patient PBMCs required for maximal isolation rates has not been rigorously established, but as few as 3×10^5 PBMCs (the number typically found in 0.3 ml of blood) is usually sufficient; culture of greater number of cells does not appreciably improve the probability of an isolate. Viral antigens or RT is usually detected after 7 to 14 days, rarely after 21 days in culture. Some isolates grow rapidly to high titer, while others grow more slowly. These in vitro differences of growth properties between strains may be related to differences in the in vivo rate of disease progression, but further data are sorely needed on this point.

Depletion of CD8-positive cells from the patient PBMC population is said to improve isolation rates, but this technique has not been widely validated and should still be considered as experimental. Some laboratories have used continuous lines of CD4-positive cells as targets, but there has been no clear demonstration of an advantage of such cell lines over heterologous normal donor PBMCs as targets. HTLV-I transformed cells or cells stably transfected with the tat-3 gene also show good sensitivity as targets. The concept that some HIV strains have inherent defects in regulatory protein production or activity warrants vigorous investigation.

HIV isolation rates are directly proportional to the stage of illness.[23] HIV can be recovered with relative ease from patients with late stage disease (essentially 100 per cent of patients), but isolation rates are typically somewhat lower among early, asymptomatic patients (20 to 60 per cent). Although not fully studied, it appears that recovery rates are good during the antibody negative "window," then decline as antibodies to the structural proteins rise.[3] HIV can often be recovered from plasma, but titers of free virus in plasma are low and isolates are obtained less often than with cocultivation of PBMCs. HIV can also be recovered from cerebrospinal fluid (CSF) in a substantial proportion of patients, even those without overt central nervous system manifestations.[7,27,59] The clinical significance of isolates from the CSF of apparently healthy individuals is at present unknown. The clinical and epidemiologic significance of scattered reports of inconsistent isolation of HIV from saliva, semen, breast milk, and other body fluids is likewise uncertain.

Although most laboratories use activated CD4-positive lymphocytes as targets to culture HIV, the monocyte/macrophage is an alternative target cell for in vitro cultures. Stimulation of PBMCs with macrophage-colony stimulating factor (M-CSF) may improve isolation rates and select for strains with relative tropism for macrophages rather than T cells.[46,49]

Virus isolation should be used cautiously as a diagnostic technique.

Falsely negative cultures are not infrequent, and patients tend to interpret a negative culture result as evidence of freedom from infection. False positive culture results are also a distinct possibility in a busy isolation laboratory. Ideally every HIV isolation laboratory should regularly receive and blindly process known positive and negative blood samples in order to ensure quality results.

DETECTION OF VIRAL ANTIGENS

Conventional "sandwich" antigen detection ELISAs have not proved to be very valuable for detection of HIV antigens in serum or plasma. Limited usefulness can be related to the relatively low signal to noise ratios obtained with positive samples (in the range of ratios of 2 × to 5 ×). Specimens from patients with late, clinically overt disease are usually positive, and specimens from normal uninfected persons are usually negative.[4,11,64,72,99] However, sera from early stage asymptomatic HIV-infected persons are also usually negative. Thus, antigen detection is not often useful to distinguish true from false reactives among persons with repeatably reactive ELISAs and reactive but nondiagnostic blots. Commercially available HIV antigen kits detect predominantly gag antigens. The antigen peak during the "seronegative window" is probably transient and is detected only infrequently, even among specimens obtained from seroconverting individuals in prospectively studied cohorts. The antigen rise in late stage disease is temporally associated with a decline in anti-gag antibodies. However, cause and effect relationships between rising antigen and declining antibodies in late stage disease are uncertain; it is not known if the antigen rise is due to failure of HIV-specific antibody production or if the decline in anti-gag antibodies is due to accelerated production of antigens and complexing of HIV antibodies out of the plasma.[64]

Disappointingly, routine testing of donated blood for HIV antigens has been unrewarding. Unequivocal positives are exceedingly rare; the yield does not offset the substantial costs.

DETECTION OF VIRAL NUCLEIC ACIDS

In situ hybridization studies, in which radiolabeled cloned HIV DNA is used to probe PBMCs, have demonstrated that cells are rarely (1 in 10^4 to 10^5) labeled. This observation suggests that only a small fraction of PBMCs are actively infected (transcribing HIV RNA) at any given time.[85] However, this technique is not sufficiently sensitive to detect HIV proviral DNA sequences, which may be stably integrated into host cell genomes at low copy numbers such as one or two copies per cell. Measurements of the number of cells showing positive signals by in situ hybridization should not be used to draw conclusions about the proportion of cells that are latently infected. In any case, in situ hybridization of blood cells is not a promising technique for diagnostic purposes.

Gene amplification by the polymerase chain reaction or PCR tech-

nique shows considerable promise.[87] In the PCR, a pair of synthetic 20 to 30 mer oligonucleotide primers, which correspond to highly conserved HIV sequences located 200 to 300 nucleotides apart, are prepared. In the presence of the primer pairs, the nucleic acids in the test specimen are subjected to successive rounds of melting and copying. (The process can be technically simplified by use of a thermostable polymerase.) If HIV DNA is present in the sample, the sequence intervening between the primer pairs is amplified geometrically. If no HIV DNA is present, little or no DNA is synthesized. Amplified HIV DNA in the reaction mixture can then be detected with a radiolabeled probe that corresponds to the center of the amplified nucleotide sequence. PCR appears to have a sensitivity superior to virus isolation, especially among patients with early stage illness.[1,62,63,75,79] In the author's limited experience, PCR has shown a sensitivity of 96 per cent among patients known to be infected and a specificity of 100 per cent among negative controls. PCR is not a simple technique at present, but efforts to automate the procedure are underway.

STAGE OF ILLNESS AND PROGNOSIS

HIV principally affects the immune system. Any rational system for disease classification or staging should reflect the extent of end-organ damage. The system used within the Department of Defense is the Walter Reed Staging Classification System, which defines six stages, one through six, that reflect progressive stages of immune impairment. This pathophysiology-based system has been described in detail elsewhere.[83] Other HIV disease classification systems, such as the CDC system, are based entirely on clinically overt manifestations and ignore critically important information that can be readily generated by the clinical pathology laboratory.

Accurate measurement of the number of CD4 lymphocytes per cubic millimeter of blood is a key measure of the stage of illness.[33,47,52,68] A severely depressed CD4 count is the single best predictor of imminent opportunistic infection, and a rise in the CD4 count is associated with the therapeutic affect of azidothymidine or other antiretroviral drugs. Patient management is markedly improved if the attending physician knows the CD4 count: immediate hospitalization and aggressive and invasive diagnostic procedures (such as bronchoscopy and lung biopsy) may be warranted in a patient with a depressed CD4 count, but conventional treatments and outpatient observation almost always suffice for the management of patients with normal CD4 counts.[82]

Quantitative measurement of the CD4 count is conventionally performed by flow cytometry. Peripheral blood leukocytes are stained with fluorescence-tagged monoclonal antibodies, and the percentage of lymphocytes stained with anti-CD4 is measured by sensors in the flow cytometer. The total number of CD4-positive lymphocytes per cubic millimeter of blood is derived by calculation: Total white bloodcells × Per cent of lymphocytes among white cells × Per cent of lymphocytes stained with CD4.

The CD4 count must be measured carefully. Several factors have been identified that contribute to imprecision of the CD4 count. (1) CD4 counts vary with a diurnal cycle, with peak values in the evening as much as double those of nadir values in the morning. (2) Technical variations in preparation, staining, and fractionating of cells by flow cytometry can be substantial; however, if appropriately standardized, the coefficient of variation (CV) of flow cytometric measurements from laboratory to laboratory can be kept below 3 per cent (3) Surprisingly, imprecision in conventional white blood cell count and differential cell counts is a major source of variability (CVs on the order of 25 per cent). (4) Acute minor viral infections can transiently lower the CD4 count[69]; a depressed count should be validated by retesting 3 months later.

Other measurements of immune dysfunction can also be associated with the stage of illness, such as blood concentrations of immunoglobulin isotypes, blood $beta_2$-microglobulin concentrations, and blood and urinary neopterine levels, but these laboratory measurements have not found wide usage among practitioners.[39,42,63,80,103] Many clinicians prefer to use the ratio CD4/CD8 lymphocytes rather than the absolute number of CD4 cells per cubic millimeter, but the CD8 count is relatively labile and devalues the information expressed by the CD4 count alone.

DIAGNOSIS OF HIV IN SPECIAL SETTINGS

Screening in Low Prevalence Populations

HIV antibody testing with intensive education and counselling of infected persons can be an important component of a comprehensive public health program for control of the HIV epidemic. In those settings in which HIV testing is not performed because of specific clinical indications, the diagnosis of HIV infection rests entirely upon the laboratory test results. As noted earlier, HIV screening whole virus lysate ELISAs cannot be used as stand alone tests, owing to the regular occurrence of falsely reactive test results. Depending on the prevalence of HIV in the population being tested, such as blood donors or civilian applicants to military service, the number of persons with falsely reactive screening ELISAs may actually be greater than the number with truly reactive ELISA results. For this reason, every laboratory that performs HIV screening ELISAs must have an automatic, preset integrated mechanism for confirmatory testing of ELISA reactive specimens. (The standard, "simplest form" HIV testing algorithm is shown in Figure 2.)

Indeed, most thoughtful directors of HIV screening programs believe that patients should not be informed of a reactive screening ELISA test result before the specimen has been exhaustively evaluated with confirmatory tests. By withholding ELISA data until a definitive laboratory diagnosis is reached, patients can be spared the needless fear and anxiety that is invariably engendered by a "positive AIDS test." Implicit in this approach is a rapid turn-around time for confirmatory testing.

The HIV testing algorithm currently used by the Department of

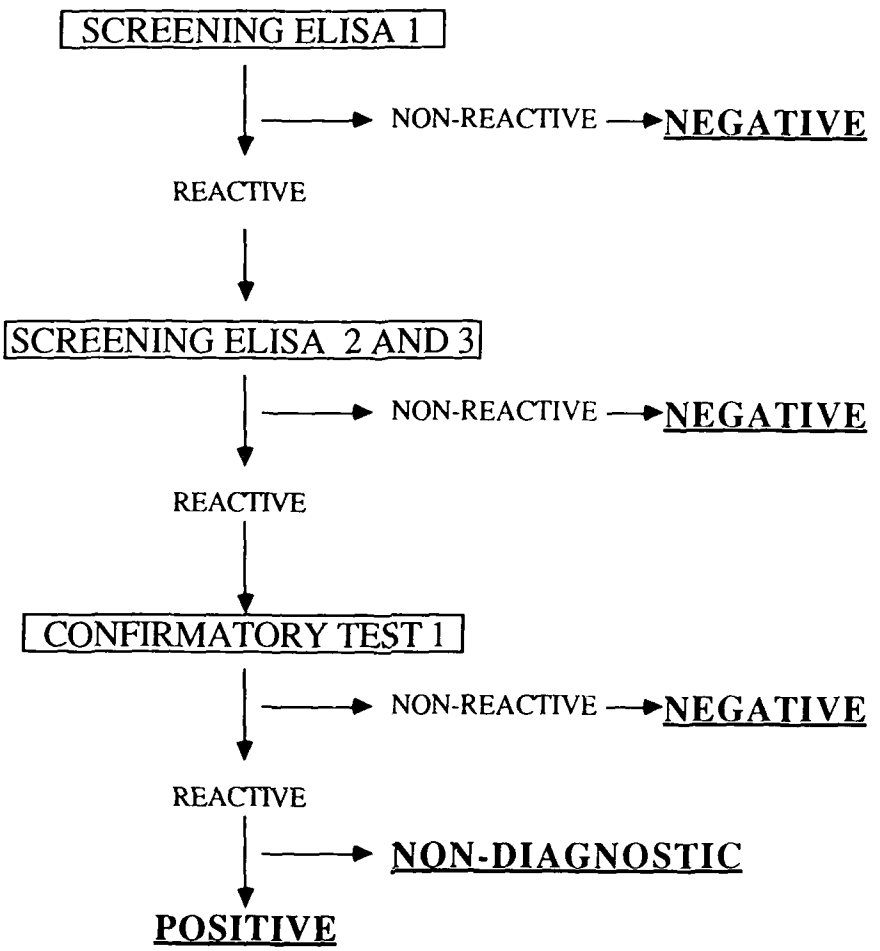

Figure 2. HIV Testing Algorithm: Simplest Form. Note that in this "simplest form" algorithm no method is used to resolve the interpretation of specimens with "nondiagnostic" results by confirmatory test, and that a second specimen is not required to verify the diagnosis.

Defense to screen civilian applicants for military service is shown in Figure 3. Several features of this algorithm deserve emphasis:

1. Screening ELISA test results are not referred to as "positive," but as reactive. Nonreactive results are reported out as negative.
2. "Reactive" screening ELISA results are not reported. Test results at this time are reported as "pending." The Western blot is the standard first confirmatory test. A clean lane, nonreactive blot is reported out as negative, and a reactive blot that meets the criteria of the ASTPHLD/DoD (see previous discussion) is reported out as positive.
3. Western blots that produce a reactive but nondiagnostic pattern are subjected to a second confirmatory test, the CBre3 molecularly cloned and expressed HIV env ELISA. Most blot reactive but nondiagnostic specimens are either clearly positive (OD > 1.0) or clearly negative (OD < 0.3) with this test.

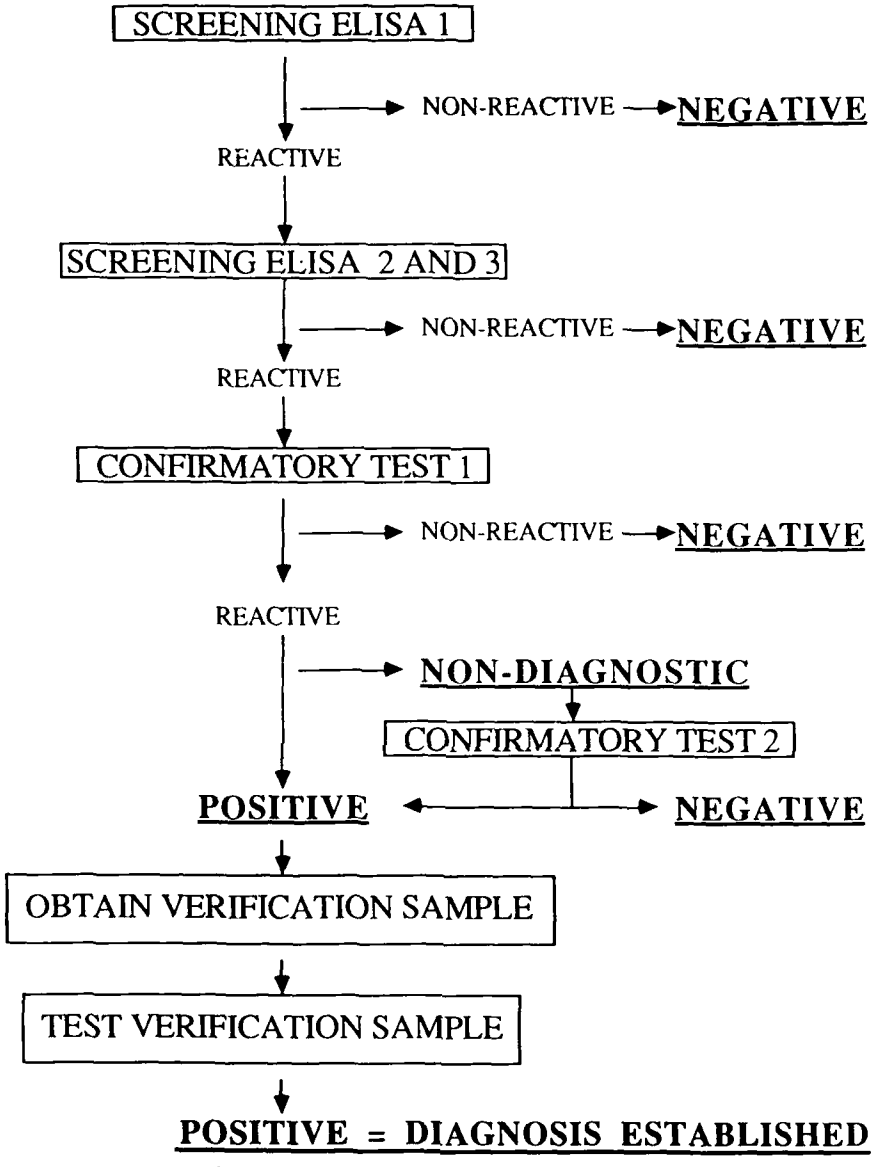

Figure 3. Complete HIV Testing Algorithm. Note that in this complete testing algorithm a second confirmatory assay is used to resolve the interpretation of specimens with "nondiagnostic" results by the first confirmatory test. For example, in US Army HIV testing programs, an immunoassay based on a molecular cloned and expressed HIV envelope polypeptide is used to resolve specimens with "nondiagnostic" or "indeterminant" results by Western blot. Also note that a new, second blood specimen must be obtained and shown to be positive before a diagnosis can be considered to be confirmed.

Rarely a specimen that is indeterminant by blot is also indeterminant by CBre3 ELISA. These cases are examined by RIPA. (Note: Some laboratories use the IFA as a second confirmatory test.) Every effort is made to avoid reporting out a result as "indeterminant."

4. If a positive diagnosis is made on the basis of testing of the first sample, the patient is advised to submit a second, new, "verification" blood sample for testing. This is done to eliminate false positive diagnoses that result from specimen handling, specimen labeling, technical, and reporting errors.

5. A diagnosis of HIV is not considered to be established unless a positive confirmatory test is recorded on two independent blood specimens from the individual.

The rate of false positive diagnoses during the first 2 years of the military applicant testing program has been retrospectively measured to be 1 in 135,000 persons tested; the predictive value of a positive diagnosis of HIV infection in the program was estimated at 99.5 per cent.[21] These results show that with careful attention to test algorithm design and with intense quality control measures, HIV testing in low prevalence populations can be extraordinarily specific.

Although the algorithm appears complex, the logistics and cost of the system are quite manageable: test results, including Western blot where applicable, are uniformly reported back to the examination station in less than 72 hours, at a cost to the government of less than $3.00 per person tested.[34]

ACUTE INFECTION

Patients who present with an illness and history compatible with acute HIV infection are a difficult challenge to the diagnostic laboratory.[58,94] At present the most certain method to establish a diagnosis is to demonstrate a seroconversion from negative to positive, but several weeks may elapse before antibodies appear. Several alternatives can be attempted, such as detection of viral antigens by sandwich ELISA, detection of viral nucleic acids by PCR, or viral culture. However, most of these techniques are either relatively insensitive or are not yet fully standardized, and conservative interpretation of results is warranted.

INFECTED BUT SERONEGATIVE PERSONS

Although most persons develop HIV antibodies within a few weeks or months after initial infection, it has been reported that some persons may remain infected but seronegative for prolonged periods, some for a year or longer.[65,78] The exact proportion of HIV-infected persons who are seronegative is impossible to determine with available diagnostic techniques. Early reports, using incompletely standardized and quality controlled culture techniques, suggested that 6 to 20 per cent of HIV-infected persons might be seronegative.[70,71,86] More recent reports, using the PCR technique, suggest that less than 1 per cent of HIV-infected adult men remain seronegative for protracted periods. Anecdotal case

reports suggest that some children infected perinatally may never develop HIV antibodies, but again the exact proportions are unknown.[19]

INFECTED INFANTS

Infants born to HIV-infected mothers present a special problem in that all such infants passively acquire HIV antibodies from their mother via transplacental transfer.[8,60] Irrespective of whether the child is infected or not, the child is strongly seropositive at birth. Maternal antibodies in the infant's circulation decline with a half-life of approximately 30 days; the uninfected child may remain seropositive throughout most of the first year of life. Special techniques must be used to establish a diagnosis. One approach is to measure synthesis of HIV antibodies by cultures of the infant's PBMCs in vitro. Alternatively, IgM antibodies to HIV have been reported detected in infant sera, but this finding has not been widely confirmed. Other approaches are to directly detect virus or viral antigen or nucleic acids.[35] HIV viral cultures, sandwich antigens assays, and PCR all show promise, but none has been thoroughly standardized for use in infants.

HIV-SPECIFIC BUT NONETHELESS FALSE POSITIVE ANTIBODY TESTS

As noted previously, infants can be strongly seropositive but nonetheless uninfected as a result of acquisition of maternal antibodies. In an analogous manner, adults can become seropositive but uninfected by parenteral inoculation with HIV antibodies contained in blood products.[40,91,100,101] Before HIV screening tests were available, most lots of hepatitis B immune globulin (HBIG) prepared from plasma pools selected from hepatitis B virus antibody positive donors were also strongly positive for antibodies to HIV. False positive antibody test results have been reported in persons who received HIV antibody positive HBIG. Most lots of immune serum globulin (ISG) produced in the United States in the early 1980s are also reactive by ELISA and Western blot, but the titers in these products are quite low, insufficient to result in seropositivity in the recipient after the ISG is functionally diluted in vivo. No cases of seropositivity have been reported to be attributable to ISG. The fractionation procedures used in the manufacture of human globulin preparations completely inactivate HIV; no cases of HIV infection have been traced to HBIG, ISG, or other human globulin preparations.

RELATED VIRUSES

Weak serologic cross-reactivity between HTLV-I and HIV-1 (HTLV-III) was reported shortly after the discovery of HIV.[9] Such cross-reactivity has not been observed with currently available conventional HIV serologic assays.

Nonetheless, other viruses more closely related to HIV-1 have been

found that do induce antibodies that cross-react in HIV-1 antibody assays. Sera from West Africans who are infected with the HIV-2 virus typically produce a reactive result when tested by HIV-1 screening ELISA.[2,31,61] HIV-1 Western blots on sera from these HIV-2-infected persons produce reactive but nondiagnostic patterns. To date only one person who is unequivocally infected with HIV-2 has been detected in the United States. Hundreds of sera from military applicants with reactive but nondiagnostic HIV-1 blots have been examined for antibodies to HIV-2; none has been positive. These results suggest that HIV-2 and viruses closely related to HIV-2 are exceedingly rare in the United States today.

CONCLUSION

In this article I have dealt exclusively with the technical issues relevant to the laboratory diagnosis of HIV. A summary of the performance characteristics of the various HIV diagnostic assays is presented in Table 3. Laboratory directors must, however, also be keenly aware of the myriad difficult and complex social, political, legal, and economic issues that profoundly affect the operations of the HIV diagnostic laboratory.[14,88] Medical and laboratory personnel must take the lead in defining, and then implementing, diagnostic and treatment procedures that

Table 3. *Laboratory Assays for Diagnosis of HIV-1 Infection: Comparison of FDA Licensure Status, Speed of Performance, and Economy*

TYPE OF ASSAY	LICENSED	SPEED*	ECONOMY†
Indirect			
Antibody Detection			
Viral lysate ELISA	Yes	4+	4+
Western blot	Yes	3+	3+
Recombinant ELISA	No	3+	4+
Immunofluorescence	No	3+	4+
Radioimmunoprecipitation	No	2+	2+
IgM immunoassay	No	3+	3+
In vitro synthesis	No	2+	1+
Functional assays	No	2+	1+
Direct			
Virus Isolation			
T-cell culture	No	1+	1+
Macrophage culture	No	1+	1+
Antigen Detection			
Sandwich ELISA	No	3+	3+
Nucleic Acid Detection			
In situ hybridization	No	2+	2+
Gene amplification (PCR)	No	2+	2+

* Rough scale of approximate time required before assay is complete and results can be reported for that assay, where 4+ = less than 4 hours; 3+ = 4 to 24 hours; 2+ = 24 to 72 hours; 1+ = more than 72 hours.

† Rough scale of approximate cost per assay in a laboratory routinely performing that assay, where 4+ = $1 per assay; 3+ = $1 to $10 per assay; 2+ = $10 to $100 per assay; 1+ = more than $100 per assay.

follow well-established traditions.[41] Politicians and lawyers can argue their social and legal agendas; medical professionals must exert leadership in defining and implementing a medical agenda based on excellence of medical care.

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